APPLICATION FOR LETTERS PATENT

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DIAGNOSTIC FOR METASTATIC PROSTATE CANCER

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DIAGNOSTIC FOR METASTATIC PROSTATE CANCER

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of International Application PCT US99/04850, filed March 2, 1999 and designating the Unites States, which application claims priority from United States Provisional Application No. 60/076,664 filed March 3, 1998.

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Not applicable.

BACKGROUND OF THE INVENTION

Prostate cancer accounts for about 1 in 10 cancer cases in the United States, and it is the most often diagnosed cancer in males [Henderson et al. (1991) *Science* 254, 1131-1138]. While in many affected patients, the tumors are slow-growing and nonmetastatic, in others the malignant prostate tumors are aggressive and metastasize. When prostate cancer metastasizes, the prognosis for the patient is poor, especially without treatment.

To date, the most frequently used test for prostate cancer is the serum level of prostate specific antigen (PSA) and the radionuclide bone scan for detecting prostate cancer metastases before definitive therapy is initiated. However, the elevated level of PSA in serum is not predictive of the pathologic stage of the prostate cancer or the presence of metastatic disease. PSA, a serine protease, is not exclusively expressed in the epithelial cells of metastatic prostate cancer, but it is also expressed in normal epithelial cells, primary tumors and benign prostate hyperplasia.

The altered expression of cell-adhesion molecules has been correlated with metastasis of many cancers. Low or no expression of E-cadherin, a cell-adhesion molecule, has been found in high-grade prostate carcinoma, and this indicates a poor prognosis for those prostate cancer patients. However, the absence of an antigen is not very useful as a diagnostic marker for cancer metastasis.

MUC18 is a glycoprotein of about 113 kDa which serves as a cell adhesion molecule on the surface of melanoma cells, and it has been correlated with the ability of melanomas to metastasize [See, e.g., Lehmann et al. (1989) *Proc. Nat. Acad. Sci. USA* 86, 9891-9895; Luca et al. (1993) *Melanoma Res.* 3, 35-41; Johnson et al. (1996) *Curr. Top. Microbiol. Immunol.* 213, 95-105; Xie et al. (1997) *Cancer Res.* 57, 2295-2303; Tang and Honn (1994-1995) *Invasion Metas.* 14, 109-122; Rummel et al. (1996) *Cancer Res.* 56, 2218-2223]. MUC18 is also known as MCAM and CD146. MUC18 carries a carbohydrate modification known as HNK-1 or CD57 [Shih et al. (1994) *Cancer Res.* 54, 2514-2520]. Besides being associated with melanoma cells' ability to metastasize, MUC18 is also associated with normal vascular tissue, and on the smooth muscle of venules, and it expresses sporadically on capillary epithelium [Johnson, J. (1994-1995) *Invasion Metas.* 14, 123-130].

There is a longfelt need in the art for an improved diagnostic test for metastatic prostate cancer so that appropriate therapy can be initiated as soon as possible and so that the number of false positive results can be minimized.

SUMMARY OF THE INVENTION

The present invention provides an improved diagnostic test for prostate cancer which has a relatively high potential for metastasis or which has metastasized. This allows the physician to choose appropriate surgical, chemotherapeutic or radiation treatment regimens. This improved assay is based on the correlation of high levels of expression of the MUC18 coding sequence as measured by MUC18 mRNA or MUC18 protein. This expression can be detected at the transcriptional level, where mRNA levels are monitored, or detection of the MUC18 gene product at the translation level can be determined, for example, through the use of an immunoassay for the MUC18 protein. The source of the material for these tests is prostate biopsy tumor tissue (e.g., from a needle biopsy) from a patient needing a

determination of the metastatic potential of a prostate tumor or from cells from a prostate tumor.

Relative levels of transcriptional expression (mRNA) of the MUC18 coding sequence can be determined by Northern hybridization analysis or by quantitative reverse transcription polymerase chain reaction (RT-PCR) in normal and neoplastic prostate tissue samples and in biopsy material.

Translational expression of MUC18 can be determined by any of a number of adaptations of an immunoassay using antibody specific for the MUC18 cell surface antigen. The relative level of MUC18 can be determined by standard immunoassays using a MUC18-specific antibody preparation and a detection system suitable for the assay. Immunoassays can include, but are not limited to, immunofluorescence assays, radioimmunoassays, enzyme-linked immunosorbent assays, and Western (immuno) blot assays. In the context of the present invention, relative amounts of the MUC18 protein are determined in tissue samples (e.g., biopsy material).

It is a further object of the present invention to provide an antibody which inhibits prostate cancer metastasis. In particular, antibody specific to MUC18 prevents metastasis of prostate cancer cells.

Additional objects include vectors directing the expression of an immunogenic fragment of human MUC18 and the corresponding recombinantly expressed protein. As specifically exemplified, an immunogenic fragment of human MUC18 is encoded by the PvuII to XhoI fragment within the sequence given in Tables 1A-1B.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the results for RT-PCR amplified human MUC18 cDNA (left panel) and cloning of the whole cDNA and its fragments into a GST-fusion protein expression vector (right panel). RT-PCR amplification of human MUC18 cDNA from the poly(A)+RNA isolated and purified from a human melanoma cell line, Sk-Mel-28. Left panel: Lane (a) shows the expected PCR product of 1957 bp (as indicated by an arrow head). Lane (m1) shows the 1 kb ladders and lane (m2) the 123-bp ladders as DNA molecular size markers. Right panel: The plasmid map of the cloned whole human MUC18 cDNA and three fragments in a GST-fusion expression system.

Fig. 2 illustrates Northern blot analysis of expression of human MUC18 in different prostate cancer cell lines. Poly(A)+RNA was isolated from human melanoma cells SK-MEL-28 (SK), human melanocyte (M), and human prostate cancer cells PC-3 (PC-3), DU145 (DU), TSU-PR-1 (TSUPR1), and LNCAP (LNCAP). The size of the human MUC18 mRNA is 3.3 kb. The amount of poly(A)+RNA (2.5 to 10 μg) is indicated as a number on top of each lane.

Fig. 3 depicts recombinant human MUC18-middle fragment. The plasmid map is shown in Fig. 1. The left panel shows the PAGE result and right panel the Superdex column purification. The GST-human MUC18 middle fragment fusion protein is shown after IPTG induction (a & b, indicated by a triangle on the left of the left panel). The fusion protein was first purified through a glutathione-Sepharose affinity column and then cleaved with the HRV-3C protease (left panel, lanes c-e). The affinity-purified recombinant human MUC18-middle fragment was then further purified through a Superdex column (right panel) to remove high molecular weight contaminants (peaks I and II, fractions 3-13). The final recombinant human MUC18-middle fragment protein is about 22 kDa (peak III, fractions 14-20), as indicated by a triangle on the right in the left panel.

Fig. 4 shows pGEX-6P, commercially available from Pharmacia Biotech, Piscataway, NJ. The specific multiple cloning site (MCS) sequences for pGEX-6P-1 (SEQ ID NO:11, encoded amino acids, SEQ ID NO:12), pGEX-6P-2 (SEQ ID NO:13, encoded amino acids, SEQ ID NO:14) and pGEX-6P-3 (SEQ ID NO:15, encoded amino acids, SEQ ID NO:16) are provided.

Fig. 5 provides diagrammatic illustrations of the whole cDNA sequence of human MUC18, its N-terminal fragment, middle fragment and C-terminal fragment, as cloned into the pGEX-6P-1 vector are provided. These fragments as cloned result in the expression of the N-terminal, middle and C-terminal fragments of the MUC18 protein. See also Tables 1A-1B for the locations of the relevant restriction sites in the cDNA sequence for human MUC18 and SEQ ID NO:1 for sequence.

Fig. 6 illustrates the results of Western blot analysis of huMUC18 protein expression in four prostatic cancer cell lines. Cellular extracts of four prostatic cancer cell lines were prepared, and the proteins were size-separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The anti-huMUC18 antibodies generated by immunization of the recombinant huMUC18-middle portion protein in chicken were used for Western blot

analysis. SK stands for cellular lysate prepared from human SK-Mel-28 cells (a), Tsu-PR1 cells (b and c), DU145 for DU145 cells (d and e), PC-3 for PC-3 cells (f and g), and LNCaP for LNCaP.FGC cells (h and i). The number over each lane indicates the amount of protein (μg) loaded in each well. The huMUC18 protein band is indicated with an open triangle. The numbers on the right-most lane indicate the protein molecular weight (kDa) rainbow markers (RPN800, Amersham).

Fig. 7 shows the results of Western blot analysis of human MUC18 protein in normal prostatic gland, normal primary human prostatic epithelial cells, and tissues of a patient with malignant prostatic cancer. Cellular lysates were prepared from normal prostatic gland (b), cultured normal primary prostatic epithelial cells (c), and prostatic cancerous tissues from a patient with malignant prostatic cancers (e-g). Cellular extracts prepared from human SK-Mel-28 cells (a) and from DU145 cells (d) were shown as the positive controls. 20μg protein of each lysate was loaded per well. The numbers on the right-most lane indicate the protein molecular weight (kDa) rainbow markers (RPN800, Amersham).

DETAILED DESCRIPTION OF THE INVENTION

Prostate cancer accounts for about 10% of all cancer cases in the United States. It is now the most frequently diagnosed cancer in American males [Rinker-Schaeffer et al. (1993) *Cancer and Metas. Rev.* 12, 3-10]. In some patients, prostate cancers metastasize rapidly, killing the patient within one year of the initial clinical presentation. In contrast, some other prostate cancer patients show a relatively slow growth of the malignant tumor without metastasis. The majority of the histologically localized prostate cancers remain subclinical and never require treatment. Prostate cancer, when truly localized, can be cured by radical prostatectomy. While in many affected patients, the tumors are slow-growing and nonmetastatic, in others prostate cancer takes a more aggressive course. Unfortunately, metastatic prostate cancer is a fatal disease without treatment.

At present, the test for serum levels of prostate specific antigen (PSA) and the radionuclide bone scan are the only diagnostic tests available before therapy is initiated. However, an elevated level of PSA observed in serum is not predictive of the pathological state of prostate cancer, nor is it correlated with metastatic prostate cancer. This is, at least in part, because PSA, a serine protease, is not exclusively expressed in the epithelial cells of

metastatic prostate cancer, but it is also expressed in normal epithelial cells, primary prostate cancerous tumors and benign prostatic hyperplasia [Wood et al. (1994) *Cancer* 74, 2533-2540]. To date, it has not been possible to predict whether histologically detected localized tumors are likely to progress to clinical cancer or when these localized tumors are likely to metastasize to other sites within the body. Thus, there is an urgent need for biochemical markers which serve to identify prostate cancers which have progressed to a stage which requires immediate surgical removal and/or additional chemotherapeutic or radiation therapeutic treatments, i.e., those cancers which are likely to metastasize.

To identify a diagnostic marker which is improved over PSA, it is crucial to understand the biochemical differences between the malignant state and the benign state and between tumor cells with high and low metastatic potential. Overexpression and underexpression of certain cell adhesion molecules at the cell surface has been proposed to reflect metastasis of several cancers [Tang and Honn (1994-1995) *Invasion Metas.* 14, 109-122]. For example, the low expression of E-cadherin has been correlated with poor prognosis of prostate cancer [Rinker-Schaeffer et al. (1993) supra]. The present invention provides a positive correlation between the level of MUC18 expression and high metastatic potential of prostate cancer cells.

The human MUC18 (huMUC18) cDNA sequence (see Table 1A) obtained by the present inventors is different from the huMUC18 cDNA sequence given in GenBank Accession No. N28882 [Johnson et al. (1994)]. The deduced amino acid sequence of huMUC18 cDNA given in Table 1A was identical to the huMUC18 sequence deposited in GenBank by Johnson's group except seven amino acid residues. This discrepancy of amino acid sequence may be due to allelic differences. However, the amino acid sequence of the inventor's huMUC18 (646 amino acids) was very different from that published by Johnson's group in 1989 (603 amino acids), which 1989 sequence appears to contain sequence errors in the huMUC18 cDNA.

The human MUC18 cDNA sequence disclosed in Table 1A was amplified by RT-PCR from poly(A)+RNA, which was isolated from the human melanoma cell line SK-Mel-28. The cDNA was cloned into the pGEM-T-easy vector (Promega, Madison, WI). The DNA sequence of the huMUC18 cDNA was determined by rapid DNA sequencing using ABI prism dye terminator cycle sequencing ready reaction kit (Perkin-Elmer) with various

huMUC18 specific primers in an automated sequencer of ABI 373XL system. The Lasergen and GCG programs were used for comparison of nucleotide and amino acid sequences of the huMUC18 cDNA.

A cDNA sequence of a human MUC18 clone has been published in GenBank under Accession No. M 28882 (See Table 1B). The translation initiation codon (ATG) is underlined, as is the translation termination codon (TAG). The PvuII (CAGCTG) and XhoI (CTCGAG) restriction sites are boxed, and cut sites are indicated by vertical arrows.

Table 1C (see also SEQ ID NO:5) shows the human MUC18 sequence as modified to introduce a BamHI site (GGATCC) just upstream of the translation start site to facilitate cloning. The translation initiation (ATG) and termination (TAG) codons are boxed. The long arrows near the 5' and

TABLE 1A

HUMAN MUC18 cDNA SEQUENCE WITH DEDUCED AMINO ACID SEQUENCE

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1    ATG GGG CTT CCC AGG CTG GTC TGC GCC TTC TTG CTC GCC GCC TGC TGC TGC TGT CCT CGC GTC GCG GGT GTG CCC GGA GAG GCT GAG CAG   90
     Met Gly Leu Pro Arg Leu Val Cys Ala Phe Leu Leu Ala Ala Cys Cys Cys Cys Pro Arg Val Ala Gly Val Pro Gly Glu Ala Glu Gln

91   CCT GCG CCT GAG CTG GTG GAG GTG GAA GTG GGC AGC ACA GCC CTT CTG AAG TGC GGC CTC TCC CAG TCC CAA GGC AAC CTC AGC CAT GTC   180
     Pro Ala Pro Glu Leu Val Glu Val Glu Val Gly Ser Thr Ala Leu Leu Lys Cys Gly Leu Ser Gln Ser Gln Gly Asn Leu Ser His Val

181  GAC TGG TTT TCT GTC CAC AAG GAG AAG CGG ACG CTC ATC TTC CGT GTG CGC CAG GGC CAG GGC CAG AGC GAA CCT GGG GAG TAC GAG CAG   270
     Asp Trp Phe Ser Val His Lys Glu Lys Arg Thr Leu Ile Phe Arg Val Arg Gln Gly Gln Gly Gln Ser Glu Pro Gly Glu Tyr Glu Gln

271  CGG CTC AGC CTC CAG GAC AGA GGG GCT ACT CTG GCC CTG ACT CAA GTC ACC CCC CAA GAC GAG CGC ATC TTC TTG TGC CAG GGC AAG CGC   360
     Arg Leu Ser Leu Gln Asp Arg Gly Ala Thr Leu Ala Leu Thr Gln Val Thr Pro Gln Asp Glu Arg Ile Phe Leu Cys Gln Gly Lys Arg

361  CCT CGG TCC CAG GAG TAC CGC ATC CAG CTC CGC GTC TAC AAA GCT CCG GAG GAG CCA AAC ATC CAG GTC AAC CCC CTG GGC ATC CCT GTG   450
     Pro Arg Ser Gln Glu Tyr Arg Ile Gln Leu Arg Val Tyr Lys Ala Pro Glu Glu Pro Asn Ile Gln Val Asn Pro Leu Gly Ile Pro Val

451  AAC AGT AAG GAG CCT GAG GAG GTC GCT ACC TGT GTA GGG AGG AAC GGG TAC CCC ATT CCT CAA GTC ATC TGG TAC AAG AAT GGC CGG CCT   540
     Asn Ser Lys Glu Pro Glu Glu Val Ala Thr Cys Val Gly Arg Asn Gly Tyr Pro Ile Pro Gln Val Ile Trp Tyr Lys Asn Gly Arg Pro

541  CTG AAG GAG GAG AAG AAC CGG GTC CAC ATT CAG TCG TCC CAG ACT GTG GAG TCG AGT GGT TTG TAC ACC TTG CAG AGT ATT CTG AAG GCA   630
     Leu Lys Glu Glu Lys Asn Arg Val His Ile Gln Ser Ser Gln Thr Val Glu Ser Ser Gly Leu Tyr Thr Leu Gln Ser Ile Leu Lys Ala

631  CAG CTG GTT AAA GAA GAC AAA GAT GCC CAG TTT TAC TGT GAG CTC AAC TAC CGG CTG CCC AGT GGG AAC CAC ATG AAG GAG TCC AGG GAA   720
     Gln Leu Val Lys Glu Asp Lys Asp Ala Gln Phe Tyr Cys Glu Leu Asn Tyr Arg Leu Pro Ser Gly Asn His Met Lys Glu Ser Arg Glu

721  GTC ACC GTC CCT GTT TTC TAC CCG ACA GAA AAA GTG TGG CTG GAA GTG GAG CCC GTG GGA ATG CTG AAG GAA GGG GAC CGC GTG GAA ATC   810
     Val Thr Val Pro Val Phe Tyr Pro Thr Glu Lys Val Trp Leu Glu Val Glu Pro Val Gly Met Leu Lys Glu Gly Asp Arg Val Glu Ile

811  AGG TGT TTG GCT GAT GGC AAC CCT CCA CCA CAC TTC AGC ATC AGC AAG CAG AAC CCC AGC ACC AGG GAG GCA GAG GAA GAG ACA ACC AAC   900
     Arg Cys Leu Ala Asp Gly Asn Pro Pro Pro His Phe Ser Ile Ser Lys Gln Asn Pro Ser Thr Arg Glu Ala Glu Glu Glu Thr Thr Asn

901  GAC AAC GGG GTC CTG GTG CTG GAG CCT GCC CGG AAG GAA CAC AGT GGG CGC TAT GAA TGT CAG GGC CTG GAC TTG GAC ACC ATG ATA TCG   990
     Asp Asn Gly Val Leu Val Leu Glu Pro Ala Arg Lys Glu His Ser Gly Arg Tyr Glu Cys Gln Gly Leu Asp Leu Asp Thr Met Ile Ser

991  CTG CTG AGT GAA CCA CAG GAA CTA CTG GTG AAC TAT GTG TCT GAC GTC CGA GTG AGT CCC GCA GCC CCT GAG AGA CAG GAA GGC AGC AGC  1080
     Leu Leu Ser Glu Pro Gln Glu Leu Leu Val Asn Tyr Val Ser Asp Val Arg Val Ser Pro Ala Ala Pro Glu Arg Gln Glu Gly Ser Ser

1081 CTC ACC CTG ACC TGT GAG GCA GAG AGT AGC CAG GAC CTC GAG TTC CAG TGG CTG AGA GAA GAG ACA GGC CAG GTG CTG GAA AGG GGG CCT  1170
     Leu Thr Leu Thr Cys Glu Ala Glu Ser Ser Gln Asp Leu Glu Phe Gln Trp Leu Arg Glu Glu Thr Gly Gln Val Leu Glu Arg Gly Pro

1171 GTG CTT CAG TTG CAT GAC CTG AAA CGG GAG GCA GGA GGC GGC TAT CGC TGC GTG GCG TCT GTG CCC AGC ATA CCC GGC CTG AAC CGC ACA  1260
     Val Leu Gln Leu His Asp Leu Lys Arg Glu Ala Gly Gly Gly Tyr Arg Cys Val Ala Ser Val Pro Ser Ile Pro Gly Leu Asn Arg Thr

1261 CAG CTG GTC AAC GTG GCC ATT TTT GGC CCC CCT TGG ATG GCA TTC AAG GAG AGG AAG GTG TGG GTG AAA GAG AAT ATG GTG TTG AAT CTG  1350
     Gln Leu Val Asn Val Ala Ile Phe Gly Pro Pro Trp Met Ala Phe Lys Glu Arg Lys Val Trp Val Lys Glu Asn Met Val Leu Asn Leu

1351 TCT TGT GAA GCG TCA GGG CAC CCC CGG CCC ACC ATC TCC TGG AAC GTC AAC GGC ACG GCA AGT GAA CAA GAC CAA GAT CCA CAG CGA GTC  1440
     Ser Cys Glu Ala Ser Gly His Pro Arg Pro Thr Ile Ser Trp Asn Val Asn Gly Thr Ala Ser Glu Gln Asp Gln Asp Pro Gln Arg Val

1441 CTG AGC ACC CTG AAT GTC CTC GTG ACC CCG GAG CTG TTG GAG ACA GGT GTT GAA TGC ACG GCC TCC AAC GAC CTG GGC AAA AAC ACC AGC  1530
     Leu Ser Thr Leu Asn Val Leu Val Thr Pro Glu Leu Leu Glu Thr Gly Val Glu Cys Thr Ala Ser Asn Asp Leu Gly Lys Asn Thr Ser

1531 ATC CTC TTC CTG GAG CTG GTC AAT TTA ACC ACC CTC ACA CCA GAC TCC AAC ACA ACC ACT GGC CTC AGC ACT TCC ACT GCC AGT CCT CAT  1620
     Ile Leu Phe Leu Glu Leu Val Asn Leu Thr Thr Leu Thr Pro Asp Ser Asn Thr Thr Thr Gly Leu Ser Thr Ser Thr Ala Ser Pro His

1621 ACC AGA GCC AAC AGC ACC TCC ACA GAG AGA AAG CTG CCG GAG CCG GAG AGC CGG GGC GTG GTC ATC GTG GCT GTG ATT GTG TGC ATC CTG  1710
     Thr Arg Ala Asn Ser Thr Ser Thr Glu Arg Lys Leu Pro Glu Pro Glu Ser Arg Gly Val Val Ile Val Ala Val Ile Val Cys Ile Leu

1711 GTC CTG GCG GTG CTG GGC GCT GTC CTC TAT TTC CTC TAT AAG AAG GGC AAG CTG CCG TGC AGG CGC TCA GGG AAG CAG GAG ATC ACG CTG  1800
     Val Leu Ala Val Leu Gly Ala Val Leu Tyr Phe Leu Tyr Lys Lys Gly Lys Leu Pro Cys Arg Arg Ser Gly Lys Gln Glu Ile Thr Leu

1801 CCC CCG TCT CGT AAG AGC GAA CTT GTA GTT GAA GTT AAG TCA GAT AAG CTC CCA GAA GAG ATG GGC CTC CTG CAG GGC AGC AGC GGT GAC  1890
     Pro Pro Ser Arg Lys Ser Glu Leu Val Val Glu Val Lys Ser Asp Lys Leu Pro Glu Glu Met Gly Leu Leu Gln Gly Ser Ser Gly Asp

1891 AAG AGG GCT CCG GGA GAC CAG GGA GAG AAA TAC ATC GAT CTG AGG CAT TAG CCCCGAAAT
     Lys Arg Ala Pro Gly Asp Gln Gly Glu Lys Tyr Ile Asp Leu Arg His End
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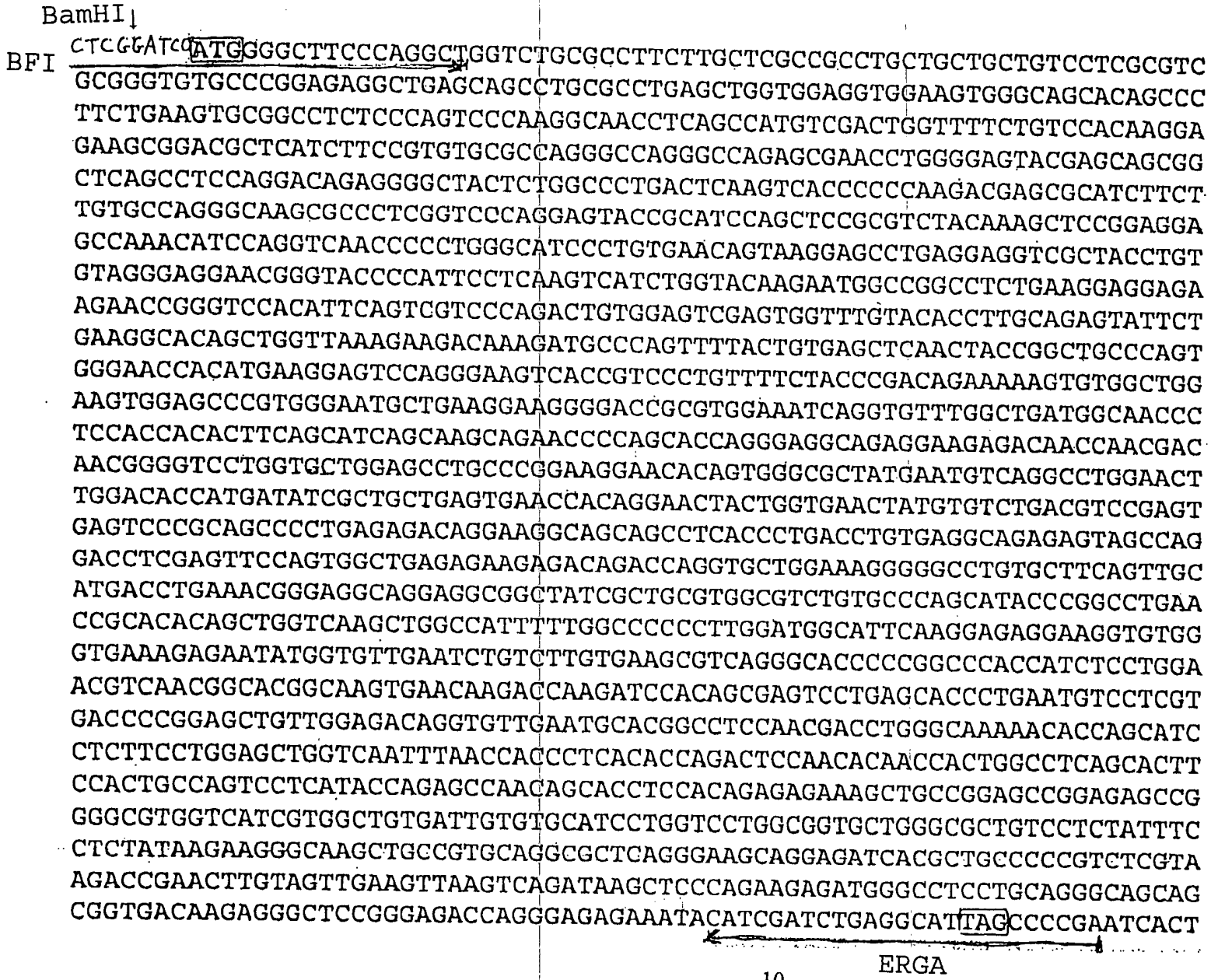
TABLE 1B

HUMAN MUC18 cDNA (GENBANK ACCESSION NO. M28882).

GGGAAGCATGGGGCTTCCCAGGCTGGTCTGCGCCTTCTTGCTCGCCGCCTGCTGCTGCTGTCCTCGCGTC
GCGGGTGTGCCCGGAGAGGCTGAGCAGCCTGCGCCTGAGCTGGTGGAGGTGGAAGTGGGCAGCACAGCCC
TTCTGAAGTGCGGCCTCTCCCAGTCCCAAGGCAACCTCAGCCATGTCGACTGGTTTTCTGTCCACAAGGA
GAAGCGGACGCTCATCTTCCGTGTGCGCCAGGGCCAGGGCCAGAGCGAACCTGGGGAGTACGAGCAGCGG
CTCAGCCTCCAGGACAGAGGGGCTACTCTGGCCCTGACTCAAGTCACCCCCCAAGACGAGCGCATCTTCT
TGTGCCAGGGCAAGCGCCCTCGGTCCCAGGAGTACCGCATCCAGCTCCGCGTCTACAAAGCTCCGGAGGA
GCCAAACATCCAGGTCAACCCCCTGGGCATCCCTGTGAACAGTAAGGAGCCTGAGGAGGTCGCTACCTGT
GTAGGGAGGAACGGGTACCCCATTCCTCAAGTCATCTGGTACAAGAATGGCCGGCCTCTGAAGGAGGAGA
AGAACCGGGTCCACATTCAGTCGTCCCAGACTGTGGAGTCGAGTGGTTTGTACACCTTGCAGAGTATTCT
GAAGGCACAGCTGGTTAAAGAAGACAAAGATGCCCAGTTTTACTGTGAGCTCAACTACCGGCTGCCCAGT
GGGAACCACATGAAGGAGTCCAGGGAAGTCACCGTCCCTGTTTTCTACCCGACAGAAAAAGTGTGGCTGG
AAGTGGAGCCCGTGGGAATGCTGAAGGAAGGGGACCGCGTGGAAATCAGGTGTTTGGCTGATGGCAACCC
TCCACCACACTTCAGCATCAGCAAGCAGAACCCCAGCACCAGGGAGGCAGAGGAAGAGACAACCAACGAC
AACGGGGTCCTGGTGCTGGAGCCTGCCCGGAAGGAACACAGTGGGCGCTATGAATGTCAGGCCTGGAACT
TGGACACCATGATATCGCTGCTGAGTGAACCACAGGAACTACTGGTGAACTATGTGTCTGACGTCCGAGT
GAGTCCCGCAGCCCCTGAGAGACAGGAAGGCAGCAGCCTCACCCTGACCTGTGAGGCAGAGAGTAGCCAG
GACCTCGAGTTCCAGTGGCTGAGAGAAGAGACAGACCAGGTGCTGGAAAGGGGGCCTGTGCTTCAGTTGC
ATGACCTGAAACGGGAGGCAGGAGGCGGCTATCGCTGCGTGGCGTCTGTGCCCAGCATACCCGGCCTGAA
CCGCACACAGCTGGTCAAGCTGGCCATTTTTGGCCCCCCTTGGATGGCATTCAAGGAGAGGAAGGTGTGG
GTGAAAGAGAATATGGTGTTGAATCTGTCTTGTGAAGCGTCAGGGCACCCCCGGCCCACCATCTCCTGGA
ACGTCAACGGCACGGCAAGTGAACAAGACCAAGATCCACAGCGAGTCCTGAGCACCCTGAATGTCCTCGT
GACCCCGGAGCTGTTGGAGACAGGTGTTGAATGCACGGCCTCCAACGACCTGGGCAAAAACACCAGCATC
CTCTTCCTGGAGCTGGTCAATTTAACCACCCTCACACCAGACTCCAACACAACCACTGGCCTCAGCACTT
CCACTGCCAGTCCTCATACCAGAGCCAACAGCACCTCCACAGAGAGAAAGCTGCCGGAGCCGGAGAGCCG
GGGCGTGGTCATCGTGGCTGTGATTGTGTGCATCCTGGTCCTGGCGGTGCTGGGCGCTGTCCTCTATTTC
CTCTATAAGAAGGGCAAGCTGCCGTGCAGGCGCTCAGGGAAGCAGGAGATCACGCTGCCCCCGTCTCGTA
AGACCGAACTTGTAGTTGAAGTTAAGTCAGATAAGCTCCCAGAAGAGATGGGCCTCCTGCAGGGCAGCAG
CGGTGACAAGAGGGCTCCGGGAGACCAGGGAGAGAAATACATCGATCTGAGGCATTAGCCCCGAATCACT

TABLE 1C

HUMAN MUC18 cDNA WITH 5' MODIFICATION TO FACILITATE CLONING



3' ends indicate primer positions (BF1 and ER6a, respectively). The cut site for BamHI within its recognition sequence (GGATCC) is indicated with a vertical arrow.

The GenBank M 28882 sequence (given in SEQ ID NO:3) is identical to a human MUC18 cDNA clone (huMUC18) from human SK-Mel-28 cells, a human malignant melanoma cell line which produces relatively high levels of the MUC18 protein. This sequence is slightly different from the huMUC18 previously published [Johnson and Rummel (1996) in *Immunology of Human Melanoma*, ed, Maio, M., IOA Press, Washington, DC, pp. 31-38; Lehmann et al. (1989) *Proc. Natl. Acad. Sci. USA* 86, 9891-9895; Luca et al. (1993) *Melanoma Res.* 3, 35-41]. The two cDNAs have three stretches of amino acid (aa) residues that are different, such as 19 aa and 17 aa at the N-terminal portion and 17 aa near the C-terminal portion. Furthermore, the published human MUC18 cDNA sequence was missing 42 amino acids at the C-terminal end (see SEQ ID NO:4). The human and murine cDNAs have 74.5% identity in the deduced amino acid sequences. The 3'-end primer used previously, ER6, did not include the last few codons and the termination codon. To re-clone the intact correct human MUC18 cDNA, a correct new 3'-primer, ER6a, for amplifying the intact human MUC18 cDNA was designed (see hereinbelow).

Efforts to express the recombinant huMUC18 protein in the pCal-n expression system (Stratagene, La Jolla, CA) in *E. coli* failed. Finally, the expression was possible by using a GST-fusion protein expression system. The huMUC18 cDNA was cloned in the PGEX-6p-1 vector (Pharmacia), a small amount of the nearly intact MUC18 protein in *E. coli* was expressed. Fortunately, the sequence of the middle portion of the huMUC18 cDNA was correct, and it was then used for making recombinant protein in *E. coli.* Only when the middle 166 amino acid portion encoded by the cDNA, but not the N-terminal or C-terminal portions, was used for expression, over-expression of the recombinant protein was possible. One pair of primers: BamHI-HMUC18-pvuII (28-mer, GGATCCCAGCTGGTTAAAGAAGACAAAG) (SEQ ID NO:6) and HMUC18-xhoI (27-mer, CTGGAACTCGAGGTCCTGGCTACTCTC) (SEQ ID NO:7) were used for PCR-amplification of the region from PvuII to XhoI of the HuMUC18 cDNA. The amplified fragment was cloned into pGEM-T Easy vector. The DNA fragment that included the coding region from the PvuII site to the XhoI site was excised from the pGEM-T Easy recombinant plasmid by two restriction enzymes, BamHI and SalI, and cloned into the BamHI and SalI

cleaved pGEX-6P-1 vector. The recombinant HuMUC18-middle fragment after cleavage with PrScission protease and purification contained the following sequences (see also Table 1A and SEQ ID NO:2).

211 376

(Gly-Pro-Leu-Gly-Ser-)(SEQ ID NO:8)**Gln-Leu-** . . . . . **Leu-Glu-Phe-Gln-**(Asn-His).

PGEX-6P-1 vector (Amino acids 211-376 of SEQ ID NO:2) pGEM T Easy vector

A 22 kDa protein fragment was expressed from the PvuII to XhoI fragment of the cDNA (see Tables 1A and 1C). A large-scale preparation of the recombinant human MUC18 "middle portion" fragment was carried out. More than 6 mg of the purified recombinant protein was obtained after purification through a glutathione-affinity column, cleavage with the HRV-3C protease, being eluted and concentrated, and further purification through a Superdex column in a Pharmacia FPLC system. After final concentration of the eluant, 6 mg of the "middle portion" recombinant protein was sent to Lampire Biological Laboratories to make polyclonal antibodies in chickens. High antibody titers were reported. Eggs are collected and IgY (chicken antibody protein) is purified from these eggs. After the titers of these purified IgY preparations are determined, they are used for immunological testing.

Using the MUC18-specific antibody preparation from chickens after immunization with the purified human recombinant huMUC18-middle fragment protein, the present inventor has shown that these antibodies can react with the human MUC18 protein expressed in human prostate cancerous cell lines and prostatic cancerous tissues by Western blot analysis. The results showed that the human MUC18 protein was only expressed in three metastatic prostate cancer cell lines (Tsu-PR-1, DU145 and PC-3), but not in one non-metastatic cell line (LNCaP.FGC). These results are consistent with the Northern blot analysis of the expression of huMUC18 mRNA in these prostatic cancer cell lines, described herein. The human MUC18 protein was weakly expressed in normal prostatic epithelial cells and in normal prostate gland, but highly expressed in human cancerous prostatic tissues, as shown by Western blot analysis of the extracts prepared from these tissues. See Fig. 6 for the results of Western blot analysis.

Further immunohistochemical analysis revealed that huMUC18 is expressed in the membrane of the expected special cell types, such as metastatic melanoma tissues, endothelial

cells, and smooth muscle cells. This indicates that the antibodies are very specific for huMUC18 antigen and work well with the formaldehyde-fixed, paraffin-embedded tissue sections. As expected, the antibodies did not react with any antigens of the normal secretory epithelial cells in the acini of the prostate gland. Interestingly, they react with the secretory epithelial cells in the acini of the prostate cancer tissues. The expression of human MUC18 protein (or antigen) only in cancerous epithelial cells, but not in normal epithelial cells, supports the use of human MUC18 as a diagnostic marker for the metastatic potential of prostate cancers.

Table 2 summarizes the results for MUC18 expression in four prostate cancer cell lines in comparison with pertinent results published by other groups. Expression of MUC18 in these cell lines is consistent with their low or no expression of E-cadherin and α-catenin and their extent of invasiveness *in vitro* and metastasis in nude mice. See Fig. 6 for Western blot analysis.

The present work has correlated relatively high levels of MUC18 with the ability of prostate cancer cells to metastasize. High levels of MUC18 expression were observed in the three metastatic prostate cancer cell lines TSU-PR1, DU145 and PC-3. MUC18 expression was not detectable in the LNCAP prostate cell line, which is not metastatic. Nonmetastatic prostate cancer cells and normal prostate cells produce no or barely detectable expression of MUC18 either as protein or mRNA. Experiments in which the LNCAP cell line is genetically engineered to express MUC18 at high levels demonstrate that when cells gain the capacity to express MUC18 at high levels, those cells gain the ability to metastasize. Experiments in which the nonmetastatic prostate cancer cell line LNCaP.FGC is genetically engineered to express MUC18 at high levels demonstrate that when the cells gain the capacity to express MUC18 at high levels, the ability to metastasize is also gained. Thus, the relative level of MUC18 expression in prostate tumor tissue is correlated with the ability to metastasize, and measurement of MUC18 expression in prostate tumor biopsy tissue allows the medical practitioner to choose the most appropriate therapy for each prostate cancer patient, with high levels of MUC18 expression mandating an aggressive treatment strategy, likely including surgery, chemotherapy and/or radiation.

TABLE 2

RELATIVE LEVELS OF MUC18 EXPRESSION

	TSU-PR1	DU145	PC-3	LNCAP	Reference
E-cadherin expression	0	0.1	0.6	1.1	Morton et al. (1993) *Cancer Res.* 53,3585-3590
α-catenin expression	none	none	none	yes	Morton et al. (1993)
Total RNA (MUC18)	yes	yes	yes	none	this work
tumor growth in nude mice	yes (2000)	yes (500)	Yes (1400)	yes (2000)	Passaniti et al. (1992) *International J. Cancer* 51,318-324
Metastasis in nude mice	yes	yes	yes	none	Lalani et al. (1992) *Cancer Metastasis Rev.* 16,29-66
Invasiveness *in vitro*	yes (220)	yes (25)	yes (20)	none	Passaniti et al. (1992)

Monoclonal or polyclonal antibodies, preferably monoclonal, specifically reacting with MUC18, may be made by methods well known in the art. See, e.g., Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratories; Goding (1986) *Monoclonal Antibodies: Principles and Practice*, 2d ed., Academic Press, New York. Also, recombinant immunoglobulins may be produced by methods known in the art, including but not limited to, the methods described in U.S. Patent No. 4,816,567. Monoclonal antibodies with affinities of 10^8 M^{-1}, preferably 10^9 to 10^{10} M^{-1} or more, are preferred.

Antibodies (polyclonal or monoclonal) specific for MUC18 are useful, for example, as probes for screening DNA expression libraries or for detecting the presence (and relative amounts) of MUC18 in a test sample, for example, prostate tumor biopsy tissue or a tissue slice of a metastatic prostate cancer, or cells in culture which were derived from a primary prostate cancerous tumor or a metastatic prostate cancer tumor. Desirably, the results are

normalized to cell number or to total cellular protein. Frequently, the polypeptides and antibodies are labeled by joining, either covalently or noncovalently, a substance which provides a detectable signal. Suitable labels include, but are not limited to, radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent agents, chemiluminescent agents, magnetic particles and the like. United States Patents describing the use of such labels include, but are not limited to, Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

Standard techniques for cloning, DNA isolation, amplification and purification, for enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like, and various separation techniques are those known and commonly employed by those skilled in the art. A number of standard techniques are described in Sambrook et al. (1989) *Molecular Cloning*, Second Edition, Cold Spring Harbor Laboratory, Plainview, New York; Maniatis et al. (1982) *Molecular Cloning*, Cold Spring Harbor Laboratory, Plainview, New York; Wu (ed.) (1993) *Meth. Enzymol.* 218, Part I; Wu (ed.) (1979) *Meth Enzymol.* 68; Wu et al. (eds.) (1983) *Meth. Enzymol.* 100 and 101; Grossman and Moldave (eds.) *Meth. Enzymol.* 65; Miller (ed.) (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Old and Primrose (1981) *Principles of Gene Manipulation*, University of California Press, Berkeley; Schleif and Wensink (1982) *Practical Methods in Molecular Biology*; Glover (ed.) (1985) *DNA Cloning* Vol. I and II, IRL Press, Oxford, UK; Hames and Higgins (eds.) (1985) *Nucleic Acid Hybridization*, IRL Press, Oxford, UK; and Setlow and Hollaender (1979) *Genetic Engineering: Principles and Methods*, Vols. 1-4, Plenum Press, New York. Abbreviations and nomenclature, where employed, are deemed standard in the field and commonly used in professional journals such as those cited herein.

All references cited in the present application are incorporated by reference herein to the extent that they are not inconsistent with the present disclosure.

The following examples are provided for illustrative purposes, and are not intended to limit the scope of the invention as claimed herein. Any variations in the exemplified articles and/or methods which occur to the skilled artisan are intended to fall within the scope of the present invention.

EXAMPLES

Example 1. Recombinant MUC18 Production and Antibody Production

The human MUC18 cDNA (1970 bp, RT-PCR amplified fragment) and three sub-fragments have been cloned in-phase in a GST-fusion protein expression system, pGEX-6p-1 (Pharmacia), which contains the glutathione-S-transferase (GST) coding region as an affinity-tag for the inserted foreign protein at its C-terminus. Fig.1 and Fig. 5 show the four possible fusions: the whole region, the N-terminal fragment, the middle fragment, and the C-terminal fragment.

Only the middle fragment of the human MUC18 protein can be induced by IPTG to express in a high amount in *E. coli* K-12 strain BL-21. Thus, only this protein is further purified for immunization. When culture A_{600} reaches 0.6 (2 to 3 hours after 1/100 inoculation of an overnight culture in L-broth with ampicillin), the expression of the recombinant middle fragment of MUC18 protein fused to GST in recombinant *E. coli* is induced by addition of 0.1 mM of IPTG to 3-liter cultures (1.5 liters per 4-liter baffled flask). Two hours after addition of IPTG at 37°C, cells are harvested by centrifugation at 3,000 rpm (2,323 x g) for 20 min in a horizontal HG-4L rotor in Sorvall RC-3 centrifuge. The cell pellet is suspended in 40 ml of ice-cold PBS (10 mM Na_2HPO4, 1.8 mM KH_2PO4, 2.7 mM KCl, and 140 mM NaCl, pH 7.3) and then lysed with a prechilled French pressure cell at 800 psi. The lysate is clarified by centrifugation for two to three times at 13,000 rpm (21,000 x g) for 30 min in SS-34 rotor in Sorvall RC-2 centrifuge. The protein concentration of the clear crude lysate adjusted to 10 mg/ml protein (about 60 ml) was used as the starting material for purification. The recombinant MUC18 proteins are purified from the clear crude lysate by batchwise adsorption to the Glutathione-Sepharose 4B affinity resin (about 20 ml of 50% slurry) by inversion on an inversion shaker at room temperature for 30 min. The GST portion of the fusion protein mediates the binding of the protein to the resin via the glutathione. After twice washing with 10 volumes (50 ml per 5 ml packed resin) of 1 X PBS and followed by twice washing with 1 X PreCission protease cleavage buffer (50 mM TrisHCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) to remove unbound proteins, the fusion protein on the resin is cleaved with 100 units of HRV-3C protease (PreCission protease, 2 units/μl, from Pharmacia) by rocking on an inversion shaker for 16 hours at 4°C. The resin was spun down at 2,000 rpm for 10 min in a Sorvall RC-32 centrifuge. The supernatant and three washings

(10 ml 1 X PBS per 10 ml resin), which contain the recombinant MUC18 protein, are then combined and concentrated by centrifuging through a Centricon-30 (Millipore/Amicon). The purity of the protein is characterized by SDS-PAGE (8 to 10% polyacrylamide gel, slab gel). The 70kDa contaminated protein is removed by passing through a Superdex 200 HR 10/30 column in 1 X PBS (void volume about 7 ml for a 20 ml packed column), and the fractions containing the recombinant middle fragment MUC18 protein (22 kDa) (eluted at about 15.5 ml) were pooled. Fig. 2 shows the SDS-PAGE results or recombinant huMUC18 protein in the GST-fusion system.

Six mg of protein is sent to Lampire Biological Lab. (Pipersville, PA) for immunizing three chickens. The anti-MUC18 antibody titers are determined by ELISA assay using the purified recombinant MUC18 as the antigen. Eggs collected during the period of high serum antibody titers are used for purification of chicken immunoglobulin IgY.

To confirm the association between MUC18 expression and metastatic ability of prostate cancer cells, the human MUC18 coding sequence is introduced into a non-metastatic, non-expressing human prostate cancer cell line (LNCAP), and clones with different levels of expression of MUC18 are isolated.

The human MUC18 cDNA has been cloned into pCR3.1 Uni (Promega, Madison, WI), a mammalian expression vector in which high levels of gene expression are driven by the human CMV-IE promoter. The human MUC18 cDNA is also cloned into a murine amphitrophic retrovirus expression vector, e.g. pZipNeoSVX [Cepko et al. (1984) *Cell* 37, 1053-1062] or LXSN [Miller and Rosman (1989) *BioTechniques* 7, 980-990], in which LTR drives gene expression.

These MUC18 recombinant vectors are used to transfect a human prostate cancer cell line which does not express MUC18, for example the LNCaP.FGC cell line [Umbas et al. (1992) *Cancer Res.* 52, 5104-5109; Iizumi et al (1987) *J. Urol.* 137: 1304-1306]. The vectors are introduced into the cultured cells by lipofection [Felgner et al. (1987) *Proc. Natl. Acad. Sci. USA* 84, 7413-7417] or by electroporation [Potter, H. (1988) *Anal. Biochem.* 174:361-373]. G418-resistant clones are selected and purified [Yuo et al. (1992) *Intervirol.* 34, 94-104] in view of the kanamycin resistance coding sequence expressed under the control of the SV40 promoter in each of the vectors. Relative expression levels of MUC18 expression in

different clones are determined by western blotting with polyclonal antibodies (described herein). Metastatic abilities are determined as described herein.

Example 2. Determination of Metastatic Ability of Prostate Cancer Cells

The degree of motility and the invasiveness of prostate cancer cells are quantitated using published methods [Tucker et al. (1994) *Eur. J. Cell Biol.* 58, 28-290; Repesh, L.A. (1989) *Invasion and Metastasis* 9, 192-208]. The Costar transwell chamber contains an inner well with a porous polycarbonate membrane, with 3 μm pore sizes in the bottom of the well. This is tightly fitted to the outer well.

To determine the motility of human prostate cancer cells, 0.5×10^5 cells are seeded in the top well. The cells remain on the top well because the poly carbonate membrane only allows medium to pass through freely. After seeding and attachment, the cells in the top well gradually migrate through the pores in the polycarbonate membrane to the bottom side of the membrane. Eventually some cells establish growth at the bottom side of the membrane. When the pore size of the membrane is about 3 μm, it somewhat slows the movement of the cells from the top side of the membrane to the bottom side. Motility of the cells is measured over the next several consecutive days. The rate of motility of a given cell line can be determined quantitatively by counting the cell number at the bottom of the membrane after trypsinization. Using this in vitro method, the motility rates of PC-3 and PC-huMUC18 human prostate cancer cells, with and without an over-expression of the human MUC18 protein, respectively, are determined and compared.

For the invasiveness of the prostate cancer cells, a similar kind of chamber is used, except before seeding the cells to the top well, the polycarbonate membrane is pre-coated with matrigel that contains protein components of the basal membranes of blood vessels. When the concentration of matrigel is correct, the membrane thus formed is thick enough to form a barrier to stop the cells from penetrating immediately, but is thin enough to allow cells to gradually invade through the membrane and migrate to the bottom of the membrane. Matrigel, which contains protein components of the base membrane of blood cell membrane, such as laminin, collagen type IV, entactin (nedogen), and heparin sulfate proteoglycan, is available commercially through Collaborative Research (Bedford, MA). Each filter in each 6.5 mm well is coated with 100 μl of a 1:20 dilution of commercial Matrigel in cold DMEM

(about 30 μg per filter). Using a similar method of counting the cells, which grow and attach to the bottom of the membrane, the rate of invasiveness of a given cell line can be quantitated [Repesh, L.A. (1989) supra]. To count the cells at the bottom of the membrane, the bottom of the membrane is treated with trypsin to detach the cells, and the cell number is counted directly using a Hemacytometer, or the cells on the membrane is stained with trypan blue and counted directly using a microscope. Alternatively, the cells are labeled with 0.6 μCi of ^{125}I-iododeoxyuridine (5 Ci/mg) for 18-24 h (about 95% of cells) and seeded to the top of the transwell chamber. After 72 h of invasion, the cells at the bottom and the cells on the top are trypsinized. The total input radioactivity is determined and compared to the radioactivities associated with cells from the top and bottom chambers. In this way the percentage of cells invading through the membrane can be accurately quantitated. Invasion rate can thus be determined [Repesh, L.A. (1989) *Invasion & Metast.* 9:192-208]. This type of in vitro test has been demonstrated to produce results in agreement with that of the in vivo animal tests [Repesh, L.A. (1989) supra]. Using this in vitro method, the invasion rates of LNCAP and LNCAP-huMUC18 transformed or transfected to express MUC18 human prostate cells, with and without over-expression of the human MUC18 protein, respectively, are determined and compared.

For comparison, the metastasis rates of these prostate cells are also tested in vivo in athymic nude mice [van Weerden et al. (1996) *Am. J. Pathol.* 149, 1055-1062]. The effect of the different expression levels of human MUC18 in prostate cancer cells on metastatic ability of different clones is determined. Similar pairs of cells as used for in vitro assay are also used in animal tests. The cells are implanted subcutaneously into nude mice. The size of tumors after different times are measured. The time and extent of the tumor cell metastases to bone or to other organs are investigated [van Weerden et al. (1996) supra].

The experiments set forth above confirm that the relative level of MUC18 expression in prostate cancer cells correlates positively with metastatic ability.

Example 3. Transcriptional Expression of MUC18

Total cell RNA is prepared from cell lines or from prostate tumor tissue with the method of one step acid-guanidinium-thiocyanate-phenol-chloroform extraction [Chomczyuski and Sacchi (1987) *Anal. Biochem.* 162, 156-159]. We have shown that this

method consistently yields good quality RNA for RT-PCR. Poly(A)+RNA is prepared from total RNA by purifying through an oligo(dT)-cellulose column [Aviv and Leder (1972) *J. Molec. Biol.* 134, 743; Wu et al. (1985) *Int. J. Biochem.* 17, 355-363].

Northern hybridization technology is used to determine MUC18 mRNA levels expressed in the four prostate cancer cell lines [Ausubel et al. (1987) *Current Protocols in Molecular Biology*, Section 4]. Total RNA is extracted from cell preparations, electrophoretically separated in a formaldehyde-impregnated agarose gel, blotted and hybridized to a ^{32}P-labeled human MUC18 cDNA [Feinberg and Vogelstein (1983) *Anal. Biochem.* 132, 6-13]. Relative levels of MUC18 mRNA are detected by radioautography. The amount of RNA loaded and transferred is estimated by the intensities of the 28S and 16S bands in each lane. Alternately, G3PDH mRNA can be estimated using a G3PDH-specific probe or 28S and 18S probes after stripping the MUC18-specific probe.

Quantitative RT-PCR methodology [Innis et al. (1990) PCR Protocols, Academic Press; Quantitative RT-PCR (1993) Methods and Applications Book #3, Clontech, Palo Alto, CA]. Human Sk-Mel-28 cells are used as positive control for this method.

Because the quantity of mRNA from a small amount of tissue is small, quantitative RT-PCR is used for quantifying MUC18 mRNA expression in tissues [Innis et al. (1990) supra]. The isolation kit from Boehringer-Mannheim (Indianapolis, IN) using magnetic beads is suitable for obtaining a small amount of poly(A)+RNA from prostate tissue. The quality of mRNA isolated with this kit is also excellent for translation and RT-PCR. The quantitative RT-PCR method is first established from using mRNA of cultured cells, as described above, and then is used for quantifying the expression of MUC18 mRNA in different prostate cancer tissues.

The RT step is standard: a 20 µl RT reaction contains 1 µg of poly(A)+RNA from the human melanoma cell line, SK-Mel-28, as template, 0.5 µg of oligo(dT)$_{16}$ as primer (Promega), 2 µl of 10X AMV-RT buffer (Promega, Madison, WI), 2 units of AMV-reverse transcriptase, 5 mM $MgCl_2$, 1 mM of dNTP mix (Promega), 1 unit of RNase inhibitor (Promega), and 50 µg/ml of acetyl BSA. The reaction was carried out at 42 to 48°C for one hour, and heated at 99°C for 5 min.

A 20 µl PCR reaction contained 2 µl of RT reaction mixture (containing the first-strain cDNA), 2 µl each of the two primers (20 pm/µl), 0.01 mM dNTPs, 1 µg of acetyl BSA,

2 μl of 10X PCR buffer with 15 mM $MgCl_2$ (Promega), and 0.5 units of Taq DNA polymerase (Promega, 5 units/μl). PCR cycles are as follows:

Hot start at 94°C 5 min, 80°C 30 sec

29 cycles of 94°C 30 sec, 64-66°C 30 sec, and 72°C 2 min

1 cycle of 94°C 30 sec, 64-66°C 30 sec, and 72°C 60 min

The sequences of the primers for amplification of the human MUC18 cDNA from poly(A)+RNA of human melanoma cell line SK-Mel-28 are as follows:

BF1 27-mer 5'-CTCGGGATCCATGGGGCTTCCCAGGCT (SEQ ID NO:9)

ER6A 25-mer 5'-TCGGGGCTAATGCCTCAGATCGATG (SEQ ID NO:10)

Example 4. Immunofluorescence Assay for MUC18

Where the anti-human MUC18 antibodies are made in chicken, fluorescence-tagged anti-chicken IgG are used for immunofluorescent staining. Tissue culture cells, or normal or cancer prostate tissue samples are fixed, and first reacted with the anti-human MUC18 antibodies, washed, and then reacted with the fluorescence-tagged rabbit anti-chicken antibodies [Umbas et al. (1992) *Canc. Res.* 52, 5104-5109]. The presence of human MUC18 on the surface of these cells or tissues readily detected using UV-fluorescence microscopy.

Human melanoma cells, Sk-Mel-28 (ATCC HTB 72), which express MUC18 a high level of MUC18 are used as the positive control. Human melanoma cells, WM115 (ATCC CRL 1675) which express no MUC18, are used as the negative control. Three human prostate cancer cell lines, LNCap.FGC, PC-3, and DU145 are available from American Type Culture Collection, Rockville, MD, as ATCC CRL 1740, ATCC CRL 1435 and ATCC HTB 81, respectively. One other cell line, TSU-PR1, isolated by Dr. Iizumi in Japan [Iizumi et al. (1987) *J. Urol.* 137, *J. Urol.* 137:1304-1306] and provided by Dr. John T. Isaacs, John Hopkins University, Baltimore, MD] was tested. TSU-PR1 cells are more metastatic than the other three cell lines [Graff et al. (1995) *Cancer Res.* 55, 5195-5199].

All four cell lines are grown as monolayer cultures in a 37°C incubator with an atmosphere of 5% CO_2. Tsu-PR1 and LNCap.FGC cells are grown in RPMI 1640 supplemented with 10% fetal bovine serum. PC-cells are grown in F12K medium with 10% fetal bovine serum. DU145 cells are grown in EMEM medium supplemented with pyruvate, extra non-essential amino acids and vitamins, and 10% fetal bovine serum.

Biopsy samples are taken, fixed and subjected to immunoassay using polyclonal antibody specific for human MUC18 described hereinabove, as described in Wood et al. (1994) *Cancer* 74:2533-2540.